# Analysis of *E. coli* K5 capsular polysaccharide heparosan

**Mellisa Ly**,
Department of Chemistry and Chemical Biology, Rensselaer Polytechnic Institute, 110 Eight Street, Troy, NY 12180, USA

**Zhenyu Wang**,
Department of Biology, Rensselaer Polytechnic Institute, 110 Eight Street, Troy, NY 12180, USA

**Tatiana N. Laremore**,
Department of Chemistry and Chemical Biology, Rensselaer Polytechnic Institute, 110 Eight Street, Troy, NY 12180, USA

**Fuming Zhang**,
Department of Chemical and Biological Engineering, Rensselaer Polytechnic Institute, 110 Eight Street, Troy, NY 12180, USA

**Weihong Zhong**,
Department of Biotechnology, College of Biological & Environmental Engineering, Zhejiang University of Technology, Hangzhou 310032, China

**Dennis Pu**,
Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, 110 Eight Street, Troy, NY 12180, USA

**Dmitri V. Zagorevski**,
Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, 110 Eight Street, Troy, NY 12180, USA

**Jonathan S. Dordick**, and
Department of Biology, Rensselaer Polytechnic Institute, 110 Eight Street, Troy, NY 12180, USA; Department of Chemical and Biological Engineering, Rensselaer Polytechnic Institute, 110 Eight Street, Troy, NY 12180, USA; Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, 110 Eight Street, Troy, NY 12180, USA

**Robert J. Linhardt**
Department of Chemistry and Chemical Biology, Rensselaer Polytechnic Institute, 110 Eight Street, Troy, NY 12180, USA; Department of Biology, Rensselaer Polytechnic Institute, 110 Eight Street, Troy, NY 12180, USA; Department of Chemical and Biological Engineering, Rensselaer Polytechnic Institute, 110 Eight Street, Troy, NY 12180, USA; Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, 110 Eight Street, Troy, NY 12180, USA

## Abstract

Heparosan is the key precursor for the preparation of bioengineered heparin, a potential replacement for porcine intestinal heparin, an important anticoagulant drug. The molecular weight (MW) distribution of heparosan produced by the fermentation of *E. coli* K5 was investigated.

Large-slab isocratic and mini-slab gradient polyacrylamide gel electrophoresis (PAGE) were used to analyze the MW and polydispersity of heparosan. A preparative method that allowed fractionation by continuous-elution PAGE was used to obtain heparosan MW standards. The MWs of the heparosan standards were determined by electrospray ionization Fourier-transform mass spectrometry (ESI-FT-MS). A ladder of the standards was then used to determine the MW properties of polydisperse heparosan samples. Unbleached and bleached heparosan produced by fermentation of *E. coli* K5 had similar number-averaged MWs ($M_N$), weight-averaged MWs ($M_W$), and MW ranges of 3,000 to 150,000 Da.

## Keywords

*E. coli* K5 capsular polysaccharide; Heparosan; Molecular weight determination; PAGE; ESI-FT-MS; Continuous-elution preparative electrophoresis

## Introduction

Heparin is a polysaccharide widely used as an anticoagulant drug [1–4]. In 2008, several lots of porcine intestinal heparin were contaminated with oversulfated chondroitin sulfate, which caused anaphylactoid reactions and over 100 deaths in patients receiving the contaminated drug [5–7]. While effective assays are being developed for the detection of known and unknown contaminants and adulterants in heparin [8–10], new highly regulated processes must be implemented in heparin production. Chemical synthesis is one approach for producing safe heparin, and it has been used to prepare a synthetic heparin pentasaccharide drug, Arixtra® [11]. However, Arixtra® holds a marginal share in the heparin market due to the high cost associated with its synthesis.

Recently, our laboratory [12] and others [13–15] have chemoenzymatically synthesized heparin and heparin analogs in small quantities by following the heparin and heparan sulfate biosynthetic pathway using cloned and expressed enzymes. Starting from *E. coli* K5 heparosan, two chemical steps and four enzymatic steps led our group to prepare a bioengineered heparin with anticoagulant properties comparable to porcine intestinal heparin [12]. The structure of the capsular polysaccharide from *E. coli* O10:K5:H4 heparosan is similar to that of desulfo-heparan sulfate [16]. Heparosan is a linear chain of repeating disaccharide units of [→4) β-D-glucuronic acid (GlcA) (1→4) *a*-D-N-acetylglucosamine (GlcNAc) (1→]n (Fig. 1). While the disaccharide composition of heparosan is known, it appears that its chain length varies depending on the fermentation conditions. For example, Manzoni et al. [16–18] reported that the extracellular K5 polysaccharide of *E. coli* contained two components with molecular weights of 16,000 Da and 1,500 Da. Volpi reported that K5 polysaccharide was 17,200 Da [19], while Vann et al. reported the MW at 20,000 Da [20]. Heparosan disaccharides to icosasaccharides [degree of polymerization (dp) of 20 monosaccharide units] obtained by a lyase treatment were recently purified by Sigulinsky et al. using SAX chromatography and analyzed by ESI-TOF-MS [21], but the number-averaged molecular weight ($M_N$), weight-averaged molecular weight ($M_W$), and polydispersity (P) of intact heparosan from *E. coli* K5 fermentation are unreported. Rapid and reliable methods for the analysis of heparosan polysaccharide from the fermentation broth are necessary to monitor its molecular weight properties, such as $M_N$, $M_W$ and P, during the preparation of this key precursor for bioengineered heparin identical to porcine intestinal heparin. In the present work, we have developed a PAGE-based method for the MW analysis of heparosan prepared by *E. coli* K5 fermentation, which can be used to assess the fermentation product.

## Experimental

### Chemicals

Electrophoresis-grade acrylamide, *N,N*-methylene-bisacrylamide, sucrose, glycine, ammonium persulfate (APS), *N,N,N',N'*-tetramethylenediamine (TEMED), and pre-cast 4–15% gradient mini-slab gels were from Bio-Rad (Hercules, CA, USA). Acetic acid, boric acid, disodium salt of ethyl-enediaminetetraacetic acid (EDTA), phenol red, and Alcian blue were from Fisher (Pittsburgh, PA, USA). All solvents were HPLC grade, and absolute ethanol was histological grade.

### Growth and harvesting of *E. coli* K5

*E. coli* strain Bi 8337/41 (O10:K5:H4) was from ATCC (Manassas, VA, USA). The culture was maintained on a medium containing glucose, ammonium, potassium phosphate monobasic, and trace elements. The 3 L fed-batch culture was performed in a 7 L autoclavable glass fermentor (Applikon, Schiedam, The Netherlands) to obtain extracellular *E. coli* K5 polysaccharide. The broth was harvested after a 55 h culture at 37°C. Bacteria were removed by centrifugation at 12,000×*g* for 10 min.

### Preparation of extracellular polysaccharide of *E. coli* K5

A 100-mL aliquot of supernatant was mixed with an equal volume of buffer A (50 mM sodium chloride, 20 mM sodium acetate, pH 4), and the pH was readjusted to 4. This mixture was loaded onto a column packed with diethylaminoethyl (DEAE) Sepharose resin (GE Healthcare, Waukesha, WI, USA), <20 mg heparosan/mL DEAE resin, and equilibrated with buffer A by gravity flow. The column was washed with 3 column volumes (CV) of buffer A, the sample was eluted with 2 CV of buffer B (1 M sodium chloride, 20 mM sodium acetate, pH 4), and the eluent was mixed with three volumes of ethanol and incubated at −20°C overnight in an explosion-proof freezer. The resulting precipitate containing crude heparosan was recovered by centrifugation at 12,000×*g* for 30 min, dissolved in water, and freeze-dried.

A bleaching step was performed on a portion of the sample to remove any remaining contaminants as follows. The lyophilized sample was dissolved in 1 M sodium chloride at a concentration of 15 g/L, and the pH was adjusted to 9.5 with 1 M sodium hydroxide. Hydrogen peroxide (30%) was added to afford a 1.5% (v/v) hydrogen peroxide solution, and the mixture was bleached overnight at room temperature. The bleached heparosan was precipitated by adding three volumes of ethanol, the precipitate was washed with 75% ethanol, centrifuged at 12,000×*g* for 30 min, and the pellet was dissolved in water and lyophilized.

### Microscale purification of heparosan from *E. coli* K5 fermentation supernatant

After bacteria were removed by centrifugation at 12,000×*g* for 10 min, 1 mL of supernatant was mixed with an equal volume of buffer A (50 mM sodium chloride, 20 mM sodium acetate, pH 4), and the pH was readjusted to 4. The solution was applied to a weak-anion exchange spin column, Vivapure D Mini H (Sartorius Stedim North America, Bohemia, NY, USA). The column was equilibrated with 400 μL buffer A (50 mM sodium chloride, 20 mM sodium acetate, pH 4) by centrifugation at 500×*g* for 5 min. Heparosan sample was loaded onto the column at 500×*g* for 5 min, washed with a total of 800 μL of buffer A, and heparosan was released with 200 μL of buffer B (1 M sodium chloride, 20 mM sodium acetate, pH 4). Three volumes of ethanol were then added to the released heparosan, and the mixture was allowed to precipitate at −20°C overnight in an explosion-proof freezer. The resulting precipitate was recovered by centrifugation (18,000×*g* for 30 min), dissolved in 1 mL of water, and lyophilized.

### NMR analysis

Purified heparosan was dissolved in 0.4 mL $D_2O$ (Sigma Aldrich, St. Louis, MO, USA), and then lyophilized three times from $D_2O$ for complete deuterium–hydrogen exchange. For NMR analysis, heparosan was dissolved in 0.3 mL 99.996% $D_2O$. ^{1}H-NMR spectra were acquired on a Brüker Ultrashield 600 MHz (14.1 Tesla) NMR instrument at 298 K.

### Continuous-elution preparative PAGE

Continuous-elution preparative PAGE was carried out as described elsewhere [22]. The total acrylamide concentration for preparative separation was selected by analyzing heparosan samples on 5%, 7.5%, 10%, and 15% mini-slab gels and comparing the results. Lower molecular weight fractions of heparosan were separated by a 15% total acrylamide (15% T) resolving gel containing 14.8% (w/v) acrylamide, 0.92% (w/v) *N,N'*-methylene-bis-acrylamide, and 5% (w/v) sucrose. All monomer solutions were prepared in resolving buffer (0.1 M boric acid, 0.1 Tris, 0.01 M disodium EDTA, pH 8.3). Stacking gel monomer solution was prepared in resolving buffer with the pH adjusted to 6 using HCl, and it contained 4.75% (w/v) acrylamide and 0.25% (w/v) *N,N'*-methylene-bis-acrylamide. A 10 cm × 7 mm diameter resolving gel column was cast from 4 mL of 10% T monomer solution containing 4 μL TEMED and 12 μl 10% APS. A stacking gel twice the volume of the sample volume was cast from 0.5 mL stacking gel monomer solution containing 0.5 μL TEMED and 30 μl 10% APS. Phenol red dye was added to the sample for visualization of the ion front during electrophoresis. The samples for electrophoresis contained 25% (w/v) sucrose.

The electrode running buffer was 1 M glycine and 0.2 M Tris (pH 9 without adjustment), and the lower chamber and elution buffer chamber were filled with resolving buffer. A peristaltic pump was set to 0.08 mL/min. The gel was subjected to electrophoresis at a constant power of 1 W for 12.5 h. Eluted fractions (240 μL) were passed above a 6,000 Da MWCO dialysis membrane supported by a frit at the bottom of the gel column. Gel-eluted fractions were analyzed on 15% T isocratic mini-slab gels (8.6 cm × 6.8 cm × 0.75 cm) and by ESI-FT-MS.

Prior to ESI-FT-MS analysis, an equal volume of 4 M sodium chloride was added to each gel-eluted fraction to disrupt any interactions of heparosan with the resolving buffer salts. After mixing with a vortex mixer, samples were thoroughly desalted using 3,000 MWCO Amicon Ultra-0.5 centrifugal filter devices (Millipore, Billerica, MA, USA), concentrated by lyophilization, and analyzed by ESI-FT-MS.

### Electrospray ionization Fourier-transform mass spectrometry

ESI-FT mass spectra were acquired at a resolution of 30,000 on an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) in the negative ionization mode using the following parameters: spray voltage 3 kV, capillary temperature 200°C, capillary voltage −15 V, tube lens −100 V; the sheath and auxiliary gas flow rates were set to 20 and 5 units respectively. Mobile phase consisting of 1:1 $H_2O$:acetonitrile with 0.2% formic acid was delivered by an Agilent 1200 nano-LC pump at a flow rate of 50 #L/min. The samples were infused through an Agilent 1200 autosampler.

### Molecular weight standards

A mixture of six purified heparosan fractions of determined molecular weights was prepared for use as a standard. Approximately 5% volumes of each 240 #L gel-eluted separated fraction used as a molecular marker were mixed together, lyophilized, and re-dissolved in 5 #L of water, to be loaded into the well with sucrose added for PAGE. This amount was sufficient for one PAGE analysis using a minigel with Alcian blue staining, and for the

large-slab gel, 20% of each fraction was used. The ladder of MW standards was used to analyze the molecular weight properties of K5 heparosan on mini-slab gradient and large-slab isocratic PAGE. A standard curve of log(MW) as a function of band migration distance was prepared and used to calculate the molecular weight properties of *E. coli* heparosan.

### Isocratic large-slab PAGE

The resolving 16 cm × 16 cm × 0.75 cm slab gels were cast from 25 mL of a 10% total acrylamide (10% T) monomer solution containing 9.66% (w/v) acrylamide and 0.33% (w/v) *N,N′*-methylene-bis-acrylamide, 125 μL 10% APS and 12.5 μL TEMED. The ten-well stacking gel was cast from 10 mL of 5% T solution with 50 μL 10% APS and 10 μL TEMED. Approximately 100 μg per well of heparosan were loaded for analysis of molecular weight properties. The gel was subjected to electrophoresis using a Protean II Xi Cell (Bio-Rad, Hercules, CA, USA) apparatus at 400 V for 1.25 h, and its inner core was cooled with cold tap water. After staining overnight in a solution of 0.5% (w/v) Alcian blue in 2% acetic acid, the gels were destained in water.

### Gradient mini-slab PAGE

Gradient PAGE analysis was performed using precast Tris-HCl gels, 4-15%, 8.6 cm × 6.8 cm × 0.75 cm (Bio-Rad, Hercules, CA, USA). Approximately 20 μg of heparosan were loaded in each well. The gel was subjected to electrophoresis at 200 V for 20 min, washed at room temperature in 10% (v/v) acetic acid and 25% (v/v) ethanol for 30 min, stained in Alcian blue solution for 1 h, and destained with several washes of 10% (v/v) acetic acid and 25% (v/v) ethanol until the gel background was clear.

Silver staining of the gradient gel was used to improve visualization of the MW ladder. The Alcian-stained gel was washed in 50% aqueous methanol for 2 h, rinsed with water for 2 h, and then soaked for 30 min in 50 mL of fresh silver staining solution, which consisted of 1 mL 4 M silver nitrate, 1 mL 7.6 M sodium hydroxide, and <2 mL ammonium hydroxide in 100 mL water. The gel was washed with water for 30 min, and developed in solution containing 100 μL of 2.5% (w/v) citric acid and 25 μL 38% formaldehyde in 50 mL water. The developing of the silver stain was stopped by adding 5% (v/v) acetic acid in 20% (v/v) aqueous methanol.

### Molecular weight analysis of K5 heparosan

Large-slab and mini-slab gradient gel images were acquired on an HP Scanjet 7400 at 600 dpi and processed for densitometry data using Un-Scan-it software (Silk Science Inc., Orem, UT, USA). These data were used to calculate the weight-averaged molecular weights ($M_W$), numberaveraged molecular weights ($M_N$), molecular weight ranges ($M_R$) and polydispersities (P) of heparosan samples from fermentation [21].

## Results and discussion

### DEAE micro-scale purification

The ^{1}H-NMR spectra of heparosan purified by gravity flow DEAE column chromatography and those of heparosan purified by anion exchange spin columns are shown in Fig. 2. Both spectra are similar to the previously published spectra for K5 polysaccharide [20], and are consistent with the structure shown in Fig. 1. The DEAE-columnpurified material is comparable in purity to the micro-scale spin-column-purified material. The sample prepared using rapid, micro-scale, spin-column purification affords heparosan with a purity of ~90%, which is sufficient to assess its molecular weight properties. Bleaching of the final DEAE-isolated heparosan did not affect the purity of the sample, as determined from NMR (data not shown), but it visibly changed the color from a tan/light brown to off-white. Spin

column purification (with or without bleaching) rapidly affords heparosan from fermentation supernatant of sufficient purity to characterize its molecular weight properties.

## Molecular weight analysis of heparosan from K5 fermentation

PAGE analysis has been demonstrated to be a useful tool for the rapid, parallel, and accurate determination of the molecular weight properties of pharmaceutical heparins [23]. Detailed information on the molecular weight properties, including its $M_W$, $M_N$, P, and molecular weight range, have not previously been reported for heparosan obtained from the fermentation of *E. coli* K5.

Molecular weight standards should ideally consist of molecules identical to those being analyzed. Thus, we undertook to prepare intact heparosan polysaccharides to use as standards. We relied on continuous-elution preparative electrophoresis to prepare a range of molecular weight standards that were sufficiently pure to be characterized by mass spectrometry.

**ESI-FT-MS**—The negative ionization mode was used throughout this study for the analysis of intact heparosan purified using continuous preparative electrophoresis because it resulted in a better signal than the positive mode (data not shown). PAGE-separated heparosan fractions contained two populations, differing by 18 mass units (Fig. 3). The first population was intact heparosan chains terminated with a glucuronic acid residue at their nonreducing end (NRE), and the second population of chains had an unsaturated uronic acid at their NRE, produced by the action of a heparosan lyase. This is consistent with previous reports of the presence of an *E. coli* bacterial phage lyase acting on heparosan as its substrate [17, 18]. Oligosaccharides containing unsaturated uronic acid residues on the nonreducing end appear more abundant in smaller oligosaccharide lengths. In the largest analyzed fraction containing dp80 oligosaccharide, unsaturated and saturated nonreducing ends are present in nearly equal amounts based on the relative abundances of ions corresponding to these chains. This is expected because lyase action should produce shorter oligosaccharide chains in higher abundance.

The monoisotopic molecular mass of unsaturated lyase products was observed for all analyzed polysaccharides from the bacterial heparosan sample. Panels B, D, and F in Fig. 3 show ESI-FT mass spectra of three representative fractions with two populations of heparosan chains: (1) terminated with a glucuronic acid residue; and (2) terminated with an unsaturated uronic acid residue, 18 mass units lower. In the mass spectra, peak envelopes corresponding to saturated chains overlap with those corresponding to H/Na exchange products of the unsaturated heparosan chains, making it difficult to identify the monoisotopic peak corresponding to the saturated heparosan chain. Thus, only the unsaturated heparosan chain masses were used for molecular weight assignment of gel-eluted fractions (Table 1).

Table 1 lists all analyzed heparosan chains with their calculated and observed molecular masses. The first peak in the peak envelope was used to calculate the monoisotopic molecular masses in each fraction. The average molecular mass of the major oligosaccharide components in each fraction was calculated based on the m/z of the most abundant ion in its respective peak envelope ("Electronic supplementary material" Table S1).

**Molecular weight standards**—Fractions prepared with continuous preparative electrophoresis using a 15% T gel consisted of 1–4 heparosan chain lengths. When used as molecular weight markers, only the major heparosan component in a given fraction was used for molecular weight calculations. In the ladder prepared from the gel-eluted FT-MS-characterized fractions, major heparosan molecular weight components with average masses

of 14,414 Da (dp76), 12,517 Da (dp66), 8,345 Da (dp44), 6,448 Da (dp34), 4,551 Da (dp24), and 3,793 Da (dp20) were used for standard calibration on the small gradient gels and large isocratic gels. The fractions for the ladder were selected based on their purity and spacing throughout the gel during PAGE. The fractions chosen for use as molecular weight markers appeared to be equally separated from one another and clearly contained one main component based on the relative abundance of ions, even if two or three other molecular components were identified by ESI-FT-MS.

**PAGE molecular weight analysis of bleached and unbleached heparosan—**
PAGE analyses were next performed on heparosan samples prepared from the supernatant of the *E. coli* K5 fermentation. Gels were stained with Alcian blue for visualization. Previous studies demonstrated that this dye binds to anionic polysaccharides in proportion to mass [24]. Unfractionated heparosan samples were analyzed on isocratic (Fig. 4) and gradient PAGE (Fig. 5). The resulting gels were stained with Alcian blue, scanned, and their digital images were analyzed with Un-Scan-It software. Molecular weight property calculations of unbleached and bleached heparosan relied on a standard curve constructed using a ladder of molecular weight standards. The number-averaged molecular weight ($M_N$), weight-averaged molecular weight ($M_W$), and polydispersity (P) were calculated based on previous methods [21]. The results obtained on a large isocratic gel versus a gradient gel are presented in Table 2.

The $M_N$ of unbleached bacterial heparosan determined in this study was 26,800 Da, which is higher than reported in the literature [16–18]. The heparosan used in this study probably differs from other previously reported number-averaged molecular weights due to the unique media and fermentation conditions used for *E. coli* growth. In some previous studies, the molecular weight measurement of heparosan relied on heparin and dextran standards, which also provided a potential source of error. Finally, molecular calculations from previous studies were not provided in sufficient detail to determine whether the reported values were number-averaged, weight-averaged or peak molecular weight. The heparosan used in this study is a highly polydisperse mixture comprising chains that contain an even number of repeating disaccharides, with chain lengths ranging from 3,000 to 150,000 Da, as determined by PAGE analysis. The $M_N$ of bleached heparosan was 25,000 Da, which is slightly lower than that of the unbleached product. The P values of both unbleached and bleached heparosan were similar.

By scanning the staining intensity within a lane, the mass of polysaccharide present as a function of migration distance is obtained in much the same way as a refractive index detector determines the mass of sample eluting from a size-exclusion chromatography column [21]. Thus, as long as the polysaccharide sample remains in the fractionation range of the gel, its molecular weight properties can be determined.

Two strategies were used when undertaking the analysis of heparosan's molecular weight properties, one involving a large-slab isocratic gel with a running time of 1.2 h, and the second using a precast mini-slab gradient gel with a running time of 0.33 h. The large-slab isocratic gel that gave superior band resolution of the lower molecular weight components of heparosan failed to allow the high molecular weight components to enter the gel (Fig. 4A). The resolution of the smaller heparosan chains on mini-slab gradient gel was reduced compared to that on the large-slab gel. However, the gradient mini-gel fractionation range was significantly broader than that of the isocratic gel, permitting all chain sizes of heparosan to enter the gel (Fig. 5A). This facilitated the analysis of highly polydisperse heparosan sample against a ladder of defined oligosaccharide standards.

Both gels provided excellent linearity for the heparosan standards of determined molecular weight when $\log(M_W)$ was plotted as a function of migration distance (Fig. 4C and 5C). While the five standards used do not cover the entire range of molecular weights, the uniform spacing of bands (Fig. 4A and B) suggest that molecular weight values outside the covered range could be accurately extrapolated, as suggested by the linear relationship observed in Fig. 4C. It is noteworthy that as the molecular weight of heparosan gets larger the spacing between adjacent bands gets smaller, limiting the resolution of the high molecular weight chains (Fig. 4A and B). This loss of resolution should not, however, adversely impact the calculation of molecular weight. Comparison of both methods demonstrated significant differences in the molecular weight properties of identical samples. Isocratic gel analysis gave an asymmetric profile (Fig. 4B) that consistently underestimated the dominant high molecular mass chains that fell outside of the fractionation range of the gel. It has been well established that isocratic gels provide superior optimal fractionation over a narrow range of molecular sizes, while gradient gels provide uniform fractionation of polydisperse mixtures but can compromise the resolution of individual components.

## Conclusions

The application of a precast gradient mini-gel, in combination with spin-column purification of heparosan from fermentation supernatant, allows for the rapid (<4 h) determination of the molecular weight properties of heparosan prepared from *E. coli* K5. This approach should offer a convenient method to screen and optimize fermentation conditions to afford heparosan with the optimal molecular weight characteristics for use in preparing bioengineered heparin.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

## References

1. Capila I, Linhardt RJ. Heparin-protein interactions. Angew Chem Int Ed Engl. 2002; 41:391–412. [PubMed: 12491369]

2. Munoz EM, Linhardt RJ. Heparin-binding domains in vascular biology. Arterioscler Thromb Vasc Biol. 2004; 24:1549–1557. [PubMed: 15231514]

3. Raman R, Sasisekharan V, Sasisekharan R. Structural insights into biological roles of protein–glycosaminoglycan interactions. Chem Biol. 2005; 12:267–277. [PubMed: 15797210]

4. Linhardt RJ, Claude S. Hudson Award address in carbohydrate chemistry. Heparin: structure and activity. J Med Chem. 2003; 46:2551–2564. [PubMed: 12801218]

5. Kishimoto TK, Viswanathan K, Ganguly T, Elankumaran S, Smith S, Pelzer K, Lansing JC, Sriranganathan N, Zhao GL, Galcheva-Gargova Z, Al-Hakim A, Bailey GS, Fraser B, Roy S, Rogers-Cotrone T, Buhse L, Whary M, Fox J, Nasr M, Dal Pan GJ, Shriver Z, Langer R, Venkataraman G, Austen KF, Woodcock J, Sasisekharan R. Contaminated heparin associated with adverse clinical events and activation of the contact system. N Engl J Med. 2008; 358:2457–2467. [PubMed: 18434646]

6. Guerrini M, Beccati D, Shriver Z, Naggi A, Viswanathan K, Bisio A, Capila I, Lansing JC, Guglieri S, Fraser B, Al-Hakim A, Gunay NS, Zhang Z, Robinson L, Buhse L, Nasr M, Woodcock J, Langer R, Venkataraman G, Linhardt RJ, Casu B, Torri G, Sasisekharan R. Oversulfated chondroitin

sulfate is a contaminant in heparin associated with adverse clinical events. Nat Biotechnol. 2008; 26:669–675. [PubMed: 18437154]

7. Guerrini M, Zhang Z, Shriver Z, Masuko S, Langer R, Casu B, Linhardt RJ, Torri G, Sasisekharan R. Orthogonal analytical approaches to detect potential contaminants in heparin. Proc Natl Acad Sci USA. 2009; 106:16956–16961. [PubMed: 19805108]

8. Zhang Z, Li B, Suwan J, Zhang F, Wang Z, Liu H, Mulloy B, Linhardt RJ. Analysis of pharmaceutical heparins and potential contaminants using (1)H-NMR and PAGE. J Pharm Sci. 2009; 98:4017–4026. [PubMed: 19642166]

9. Rudd TR, Skidmore MA, Guimond SE, Holman J, Turnbull JE, Lauder RM, Fernig DG, Yates EA. The potential for circular dichroism as an additional facile and sensitive method of monitoring low-molecular-weight heparins and heparinoids. Thromb Haemost. 2009; 102:874–878. [PubMed: 19888522]

10. Liu H, Zhang Z, Linhardt RJ. Lessons learned from the contamination of heparin. Nat Prod Rep. 2009; 26:313–321. [PubMed: 19240943]

11. Toschi V, Lettino M. Fondaparinux: pharmacology and clinical experience in cardiovascular medicine. Mini Rev Med Chem. 2007; 7:383–387. [PubMed: 17430224]

12. Zhang Z, Mccallum SA, Xie J, Nieto L, Corzana F, Jimenez-Barbero J, Chen M, Liu J, Linhardt RJ. Solution structures of chemoenzymatically synthesized heparin and its precursors. J Am Chem Soc. 2008; 130:12998–3007. [PubMed: 18767845]

13. Lindahl U, Li JP, Kusche-Gullberg M, Salmivirta M, Alaranta S, Veromaa T, Emeis J, Roberts I, Taylor C, Oreste R, Zoppetti G, Naggi A, Torri G, Casu B. Generation of "neoheparin" from *E. coli* K5 capsular polysaccharide. J Med Chem. 2005; 48:349–352. [PubMed: 15658847]

14. Peterson S, Frick A, Liu J. Design of biologically active heparan sulfate and heparin using an enzyme-based approach. Nat Prod Rep. 2009; 26:610–627. [PubMed: 19387498]

15. Kuberan B, Beeler DL, Lech M, Wu ZL, Rosenberg RD. Chemoenzymatic synthesis of classical and non-classical anticoagulant heparan sulfate polysacchrides. J Biol Chem. 2003; 278:52613–52621. [PubMed: 14519763]

16. Blundell CD, Roberts IS, Sheehan JK, Almond A. Investigating the molecular basis for the virulence of Escherichia coli K5 by nuclear magnetic resonance analysis of the capsule polysaccharide. J Mol Microbiol Biotechnol. 2009; 17:71–82. [PubMed: 19401610]

17. Manzoni M, Bergomi S, Cavazzoni V. Extracellular K5 polysaccharide of Escherichia coli: production and characterization. J Bioact Compat Polym. 1993; 8:251–257.

18. Manzoni M, Bergomi S, Rollini M. Influence of the culture conditions on extracellular lyase activity related to K5 polysaccharide. Biotechnol Lett. 2000; 22:81–85.

19. Volpi N. Purification of the Escherichia coli K5 capsular polysaccharide and use of high-performance capillary electrophoresis to qualitative and quantitative monitor the process. Electrophoresis. 2004; 25:3307–3312. [PubMed: 15472951]

20. Vann WF, Schmidt MA, Jann B, Jann K. The structure of the capsular polysaccharide (K5 antigen) of urinary-tract-infective Escherichia coli 010:K5:H4. A polymer similar to desulfoheparin. Eur J Biochem. 1981; 116:359–364. [PubMed: 7018909]

21. Sigulinsky C, Babu P, Victor XV, Kuberan B. Preparation and characterization of (15)N-enriched, size-defined heparan sulfate precursor oligosaccharides. Carbohydr Res. 2010; 345:250–256. [PubMed: 19945695]

22. Laremore TN, Ly M, Solakyildirim K, Zagorevski DV, Linhardt RJ. High-resolution preparative separation of glycosaminoglycan oligosaccharides by polyacrylamide gel electrophoresis. Anal Biochem. 2010 doi:10.1016/j.ab.2010.03.004 (in press).

23. Edens RE, Al-Hakim A, Weiler JM, Rethwisch DG, Fareed J, Linhardt RJ. Gradient polyacrylamide gel electrophoresis for determination of molecular weights of heparin preparations and low-molecular-weight heparin derivatives. J Pharm Sci. 1992; 81:823–827. [PubMed: 1328601]

24. Grant AC, Linhardt RJ, Fitzgerald GL, Park JJ, Langer R. Metachromatic activity of heparin and heparin fragments. Anal Biochem. 1984; 137:25–32. [PubMed: 6203432]
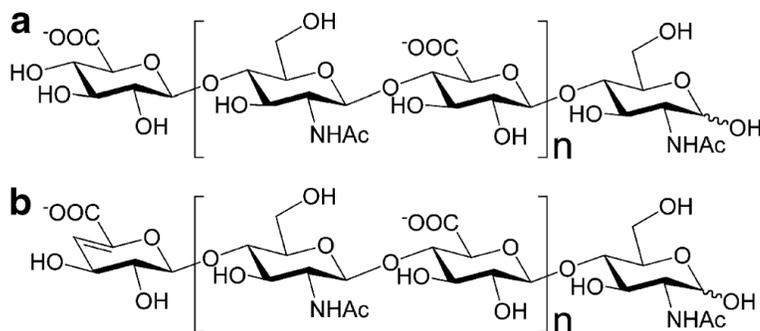
**Fig. 1.**
Structure of heparosan. **A** The structure of a native chain of heparosan that has an even number of saccharide units, with GlcNAc at the reducing end and GlcA at the nonreducing end. **B** The structure of a heparosan chain following the action of bacterial phage heparosan lyase, with a GlcNAc at the reducing end and an unsaturated uronic acid at the nonreducing end. $M_N$(bacterial heparosan) = ~26,800 Da, with $n_{avg}$ = ~70 (dp140). $M_R$ = ~3,000 Da to ~150,000 Da. Bacterial heparosan is a mixture of A and B
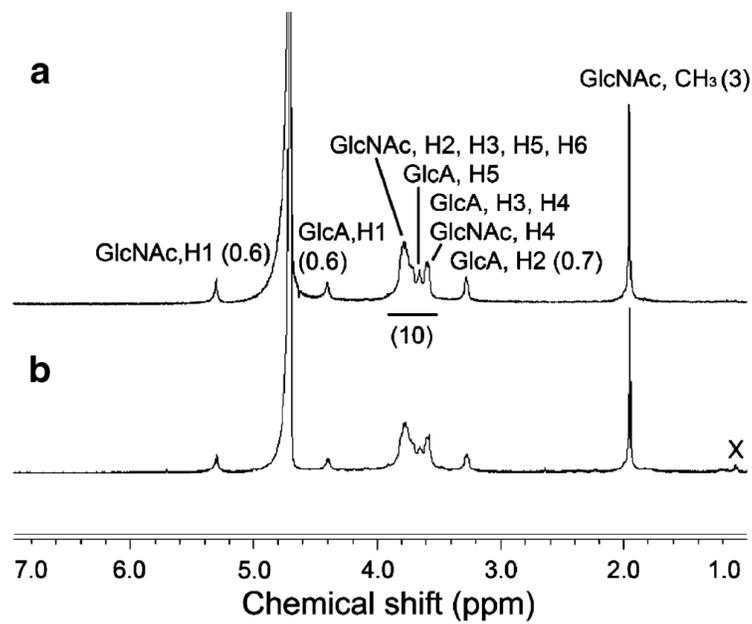
**Fig. 2.**
^{1}H-NMR (600 MHz) of heparosan. **A** Heparosan purified by DEAE column chromatography. **B** Heparosan purified by spin column chromatography (Vivapure D Mini H). An impurity is marked with an "x" near 1 ppm. Integration values are in parentheses

**Fig. 3.**
ESI-FT-MS analysis of selected heparosan fractions from continuous-elution preparative
PAGE. **A** Heparosan dp20, **C** heparosan dp54, and **E** heparosan dp80. **B**, **D**, **F** Isotopic peak
envelopes of dp20, dp54, and dp80 corresponding to chains with unsaturated and saturated
NREs are separated by $m/z$ 9, 3.6, and 3 (18 mass units), respectively
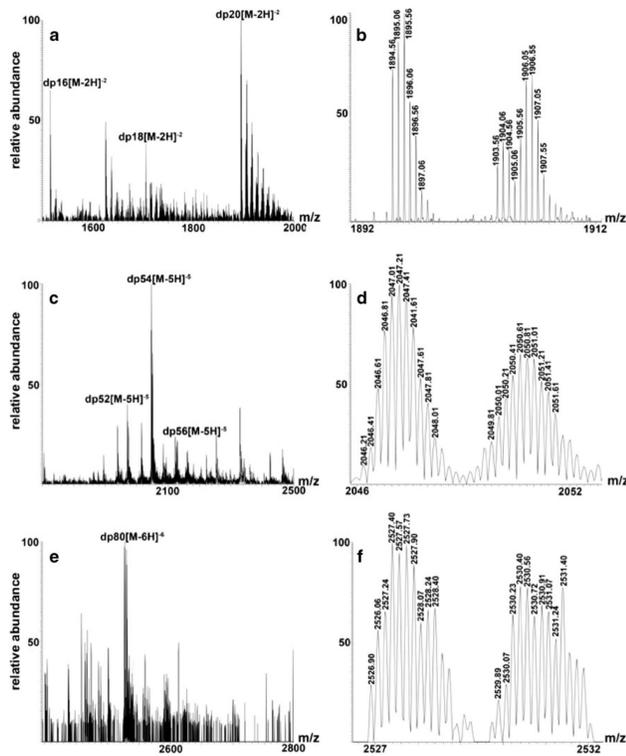
**Fig. 4.**
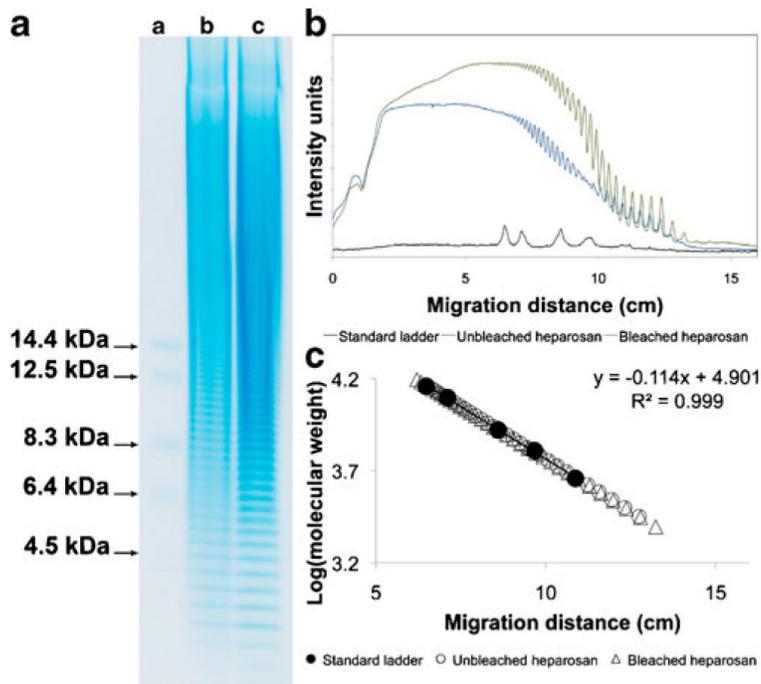Large isocratic PAGE characterization of the molecular weight properties of spin-column purified heparosan. **A** Gel stained with Alcian blue. *Lane a*: molecular weight markers (indicated with *arrows*); *lane b*: unbleached heparosan; *lane c*: bleached heparosan. **B** Scanned gel showing staining intensity as a function of migration distance. **C** Standard curve of log($M_W$) as a function of migration distance for molecular weight standards. Lining up the identified bands from standards with the well-separated bands allowed a wider gel calibration range. Note that the stacking gel (not shown) also contained Alcian-stained sample that did not enter the resolving gel
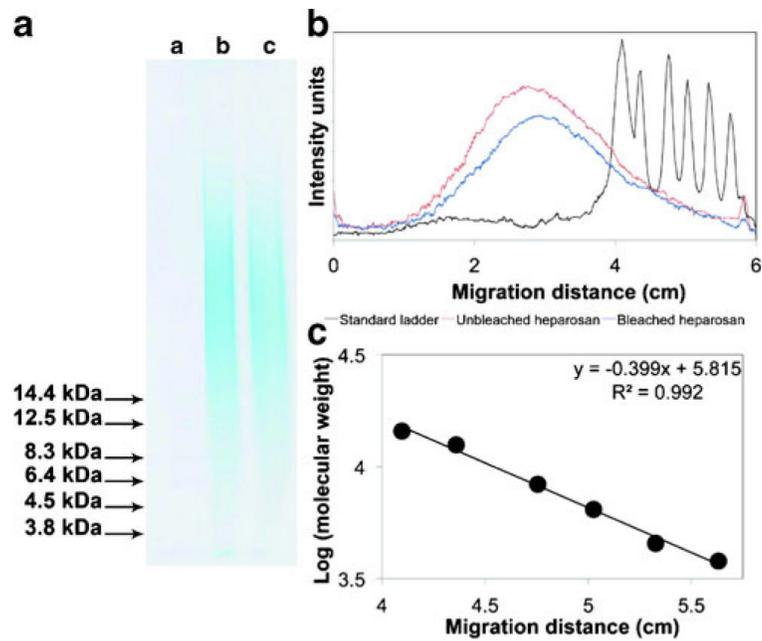
**Fig. 5.**
Mini gradient PAGE for the characterization of the molecular weight properties of spin-column purified heparosan. **A** PAGE gel stained with Alcian blue. *Lane a*: molecular weight markers (indicated with arrows); *lane b*: unbleached heparosan; *lane c*: bleached heparosan. **B** Scanned gel showing staining intensity as a function of migration distance. A silver stained ladder was used for the densitometry scan. **C** Standard curve of log($M_W$) as a function of migration distance for molecular weight standards

**Table 1**

Molecular weights of heparosans of different lengths unsaturated at the nonreducing end. ESI-FT-MS spectral ions identified oligosaccharides from continuous-elution preparative PAGE fractions based on their monoisotopic molecular mass ion and their average molecular mass *m/z* (also see the " Electronic supplementary material" Table S1)

| Degree of polymerization | Monoisotopic mass | Calculated *m/z* | Observed *m/z* | *z* | Error (ppm) |
|---|---|---|---|---|---|
| 16 | 3032.89 | 1515.44 | 1515.44 | 2 | 0 |
| 18 | 3412.00 | 1705.00 | 1704.99 | 2 | −5.9 |
| 20 | 3791.11 | 1894.56 | 1894.55 | 2 | −5.3 |
| 22 | 4170.23 | 2084.12 | 2084.11 | 2 | −4.8 |
| 24 | 4549.34 | 2273.67 | 2273.55 | 2 | −53.0 |
| | | 1515.45 | 1515.44 | 3 | −6.6 |
| 26 | 4928.45 | 2463.23 | 2464.22 | 2 | −4.1 |
| | | 1641.82 | 1641.81 | 3 | −6.1 |
| 32 | 6065.38 | 1515.35 | 1515.45 | 4 | +66.0 |
| 34 | 6444.89 | 2147.30 | 2147.30 | 3 | 0 |
| | | 1610.22 | 1610.23 | 4 | +6.2 |
| 36 | 6824.01 | 1705.00 | 1705.00 | 4 | 0 |
| 38 | 7203.12 | 1799.78 | 1799.78 | 4 | 0 |
| 40 | 7582.23 | 1894.56 | 1894.56 | 4 | 0 |
| 42 | 7961.34 | 1989.34 | 1989.35 | 4 | +5.0 |
| 44 | 8340.45 | 2084.11 | 2084.12 | 4 | +4.8 |
| | | 1667.09 | 1667.09 | 5 | 0 |
| 46 | 8719.56 | 2178.89 | 2178.91 | 4 | +9.2 |
| 48 | 9098.68 | 2273.67 | 2273.67 | 4 | 0 |
| 50 | 9477.79 | 2368.45 | 2368.46 | 4 | +4.2 |
| 52 | 9856.90 | 1970.38 | 1970.39 | 5 | +5.1 |
| 54 | 10236.01 | 2046.20 | 2046.21 | 5 | +4.9 |
| 56 | 10615.12 | 2122.02 | 2121.99 | 5 | −14.1 |
| 58 | 10994.23 | 2197.84 | 2197.95 | 5 | +50.0 |
| 60 | 11373.34 | 1894.56 | 1894.56 | 6 | 0 |
| 62 | 11752.46 | 2349.49 | 2349.50 | 5 | +4.3 |
| | | 1957.74 | 1957.75 | 6 | +5.1 |

| Degree of polymerization | Monoisotopic mass | Calculated $m/z$ | Observed $m/z$ | $z$ | Error (ppm) |
|---|---|---|---|---|---|
| 64 | 12131.57 | 2425.31 | 2425.49 | 5 | +74.2 |
| 66 | 12510.68 | 2501.14 | 2501.13 | 5 | −4.0 |
| | | 2084.11 | 2084.11 | 6 | 0 |
| 68 | 12889.79 | 2576.96 | 2577.14 | 5 | +69.8 |
| | | 2147.30 | 2147.28 | 6 | −9.3 |
| 70 | 13268.90 | 2652.78 | 2652.79 | 5 | +3.8 |
| 72 | 13648.01 | 2273.67 | 2273.70 | 6 | +13.2 |
| 74 | 14027.12 | 2336.85 | 2337.17 | 6 | +137.0 |
| 76 | 14406.24 | 2400.04 | 2400.37 | 6 | +137.5 |
| 78 | 14785.35 | 2463.23 | 2463.39 | 6 | +65.0 |
| 80 | 15164.46 | 2526.41 | 2526.90 | 6 | +194.0 |

**Table 2**

Calculated molecular weight properties ($M_W$, $M_N$, and P) of unbleached and bleached heparosan isolated from *E. coli* K5 fermentation supernatant

|         | Isocratic gel | Gradient gel |
| ------- | ------------- | ------------ |
|         | *Unbleached heparosan* | |
| $M_N$   | 14,700        | 26,800       |
| $M_W$   | 24,100        | 57,500       |
| P       | 1.64          | 2.15         |
|         | *Bleached heparosan* | |
| $M_N$   | 12,900        | 25,000       |
| $M_W$   | 21,000        | 51,500       |
| P       | 1.63          | 2.06         |